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Effects of natural current pH variability on the sea urchin *Paracentrotus lividus* larvae development and settlement

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ABSTRACT

One of the most important environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates is ocean pH. In the last decade, the effects of decreasing ocean pH as a result of climate change processes (i.e. ocean acidification) on marine organisms have been target of much research. However, the effects of natural pH variability in the species' niche have been largely neglected. Marine coastal habitats are characterized by a high environmental variability and, in some cases, organisms are already coping with pH values predicted by the end of the century. It is thought that because of adaptation or acclimation to natural environmental variability, intertidal species may have some resilience to future changes. In this study, we explored the sensitivities of the sea urchin *Paracentrotus lividus* during its larvae development and settlement undergoing two different daily pH frequencies (12 h fluctuation from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH) that have been currently recorded in the sampling region (Canary Islands). Results showed that, despite larvae development was slightly enhanced by moderated fluctuating pH regimes, *P. lividus* larva was able to develop normally in both, fluctuating and constant, pH environments. Results of the settlement experiment showed very clear patterns since postlarvae settlement was only successful when a covering of algae was added, regardless of the pH fluctuation applied.

1. Introduction

Marine coastal habitats are characterized by a high environmental variability. Especially in temperate regions, physico-chemical parameters such as temperature, pH, salinity, dissolved oxygen, turbidity, and other factors, suffer a wide variation that occurs on several spatial and temporal scales. These factors determine the features of coastal ecosystems and its variability will be the result of complex interactions between physical, chemical and biological processes (e.g. biological activity, currents, tidal excursions, subjacent oceanographical features, freshwater inputs, upwellings, etc.). Variations in physico-chemical parameters are therefore highly site-dependent with differences in their amplitude and frequency (Henson et al., 2010).

pH is, beside temperature, one of the most important environmental factors controlling the distribution, physiology, morphology and behaviour of marine invertebrates (Doney et al., 2009). In the last decade, the effects of decreasing ocean pH as a result of climate change processes (i.e. ocean acidification, OA) on marine organisms have been target of much research (see reviews by Byrne et al. 2013a,b; Byrne and Przeslawski, 2013; Dupont and Thorndyke, 2013). Most laboratory experiments have been focused on expected future values of pH, pCO_2 and saturation states for calcite and aragonite for the near future. However, it has been reported that pH in the oceans is not constant, temporal and spatial variations exist (Hofmann et al., 2011), and this natural pH variability effects on species' niche has been neglected in experimental studies. Although the levels of pH in a given environment may vary considerably, accurate records of this parameter are only very recent and/or fragmented (Dorey et al., 2013). Variation in seawater pH is higher in shallow temperate coastal environments due to their inherent ambient heterogeneity and photosynthetic activity (Middelboe & Hansen 2007). A pronounced 24 h pH cycle has been recorded in coastal environments, spanning an average of 0.24 pH units during a day (Wootton et al., 2008), although this variation depends on numerous parameters and differs at different environments. For instance, Hofmann et al. (2011) recorded high pH variability in coastal temperate sites such as estuaries, near-shore habitats and kelp forest environments of up to 0.99, 0.50 and 0.54 pH units, respectively. In shallow coastal environments the surface seawater pCO_2 is already at levels significantly higher and pH lower than expected values from equilibrium with current atmospheric levels (Fagan and Mackenzie, 2007; Bates

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et al., 2010; Thomsen et al., 2010; Shamberger et al., 2011; Yu et al., 2011; Hofmann et al., 2011).

Marine invertabrates, including calcifiers, can thrive in markedly heterogeneous environments. Thus, we have to highlight the importance of understanding species' niche to define their sensitivity to environmental changes. It is thought that species that naturally experience a high environmental variability, such as intertidal and shallow water organisms and species with a broad latitudinal distribution, may be more resilient to environmental changes than those species from relatively invariable habitats (Melzner et al., 2009; Talmage and Gobler, 2009, 2011; Moulin et al., 2011; Matson et al., 2012; Wolfe et al., 2013; Byrne et al. 2013a,b). Natural variability may occur at rates much higher than the rate at which carbon dioxide is decreasing ocean pH as a result of climate change processes (Dore et al., 2009; Byrne et al., 2010). Therefore, organisms from natural low pH and fluctuating habitats may already be experiencing pH values forecasted for the end of this century (Kelly and Hofmann, 2013). Variability in pH could potentially promote acclimation or adaptation to acidification through repeated exposure to low pH conditions; alternatively, transient exposures to high pH conditions could buffer the effects of acidification by momentary relieving physiological stress (Hofmann et al., 2011). In this sense, natural fluctuation in pH may improve the resilience of marine populations. On the contrary, OA could shift such natural variations towards even lower pH levels, making these heterogeneous environments more vulnerable to near future climate change scenarios. In addition, some studies show that some intertidal invertebrates are already living near their physiological tolerance limits (Tomanek, 2008; Somero, 2010), and shifting of baseline environmental conditions may push extremes to suboptimal or lethal levels. Therefore, the combination of a wide natural variability with the steady effects of acidification could produce extreme events with large impacts (Joint et al., 2011).

This study focused on studying larvae development and settlement of the sea urchin *Paracentrotus lividus* under fluctuating regimes of seawater pH. This species is widely distributed, and is known to cross latitudes, throughout the Mediterranean Sea and the NE Atlantic Ocean from Ireland to the Canary Islands, and habitats, from the intertidal to the subtidal. In the Canary Islands, *P. lividus* is found from the lowest intertidal, where it most commonly occupies crevices in tide-pools, to around 10 m depth in the subtidal. In the Archipelago, this sea urchin mainly inhabits environments with a dense algal cover where *Cystoseira abies-marina* is the dominant species (Girard et al., 2012). This typical habitat of the species is characterized by a wide daily pH oscillation with minimum values occurring early in the morning and maximum records in the evening, mainly due to the biological activity of abundant benthic photosynthetic organisms (Hernández et al., 2015). This daily cycle of seawater pH variation is readily explained by daily variations in photosynthesis and respiration, as well as in seawater temperature. Seawater pH increases when CO_2 is captured by photosynthetic organisms (macroalgae and phytoplankton) during the day and decreases during the night when respiration and diffusion from/to the atmosphere regulate CO_2 (Bensoussan and Gattuso, 2007). The special nature of the volcanic archipelago of the Canary Islands result in a variety of coastal habitats that experience huge environmental variability in a limited space. The planktonic larvae stage of *P. lividus* is estimated to last roughly 1 month and then settlement occurs (Girard et al., 2008). Larvae of many benthic species display active habitat selection mechanisms by responding to abiotic environmental or chemicals cues of different sources, such as conspecifics, host plants, preys, or surface-associated bacterial communities (biofilms) (Dorey, 2013). Macroalgae and their associated biofilms have been appointed as one of the main inducers producing effective cues to trigger metamorphosis and settlement of sea urchins (Pearce and Scheibling, 1990; Swanson et al., 2006).

In this study we explored the sensitivities of *P.lividus* during its larvae development undergoing two different daily pH frequencies that are currently taking place in the sampling region (Hernández et al., 2015): a 12 h fluctuation of seawater pH from 7.7 to 8.1 *vs.* a constant pH regime of 8.1 units (which is normally used as a control for experimental trials). We also tested whether pH fluctuations *per se* or any other cue related with the algae *Cystoseira abies-marina* induce settlement.

2. Material and methods

2.1. Animal collection and spawning

Mature *P. lividus* specimens (test diameter > 24 mm) were collected by scuba divers from subtidal rocky shores between 5 and 10 m depth. Individuals were collected in November of 2013 at the south coast of Tenerife Island (28°5′57″N, 16°36′53″W), during the spawning period known for the species (Girard et al., 2012).

Animals were induced to spawn by injection of 2 ml of KCl (0.5 M) through the peristomial membrane. Five males and six females, randomly selected in order to reduce experimental variability (Evans and Marshall, 2005), were used for fertilization and sexual products mixed before putting gametes of each sex in contact. Sperm was collected dry and kept on ice until usage. Eggs were collected in filtered seawater (FSW). Fertilization was done in a proportion of 1:1500 (eggs:sperm). Cleaving embryos (two cell stage) were placed at a density of 15 individuals mL^{-1} in 20 L aquaria filled with FSW and constantly aerated.

2.2. Experimental design and sea water chemistry

When the embryos reached the gastrula stage, larvae were distributed into 2 L culture beakers at densities of 5 larvae mL^{-1}. Six culture beakers were maintained in a seawater table to keep temperature conditions. Seawater was replaced in each beaker each 24 h. At day 4 post-fertilization, larvae were fed with red algae *Rhodomonas lens* at a concentration of 2000 cells mL^{-1}. The microalgae strain was provided by Spanish Oceanography Institute and cultured in the laboratory with enriched F/2 medium (Guillard and Ryther, 1962) at 20 °C and a cycle 24 h/0 h light/dark. The tested exposure pH levels within the experiments did not have effect on algal growth (Dupont et al., 2012). Algae were separated from the growth medium by centrifugation and then suspended in fresh FSW before usage. The seawater inside the culture beakers was constantly aerated and homogenized using a paddle system (Strathmann, 1987) that was moved by a micromotor keeping food and larvae in suspension.

Larvae of *P. lividus* were raised in two different treatments of pH (n = 3 for each treatment). The cultures were maintained at a salinity 36.7 and a temperature of 19 °C, corresponding to the natural conditions of seawater during November at the collection site. Larvae were cultured at contrasting pH fluctuation frequencies over a period of one month: 12 h fluctuation from 7.7 to 8.1 units of pH (Treatment 1, T1), and constant pH treatment of 8.1 units of pH (Treatment 2, T2), corresponding to the present variability of productive (high benthic macroalgae cover) and unproductive environments (low benthic macroalgae cover), respectively, at the sampling region (Hernández et al., 2015) (Fig. 1).

In order to control seawater pH, we used a computerised control system (AquaMedic) that regulated pH by bubbling pure CO_2 directly into the water to a resolution of ± 0.01 pH units. Monitoring of temperature, pH_{NBS} (Metrohm mobile meter with a Primatrode NTC IP pH electrode and temperature sensor) and salinity (handheld conductivity meter COND 315i) was performed daily. Seawater total alkalinity (TA) was measured for each treatment by titration. Other parameters of the seawater carbonate chemistry (pCO_2, calcite saturation state (Ωc) and aragonite saturation state (Ωa)) were calculated from TA and pH using CO2sys (Lewis and Wallace, 1998). Calculations were based on a set of constants K1 and K2 from Mehrbach et al. (1973) (refit by Dickson and Millero, 1987).

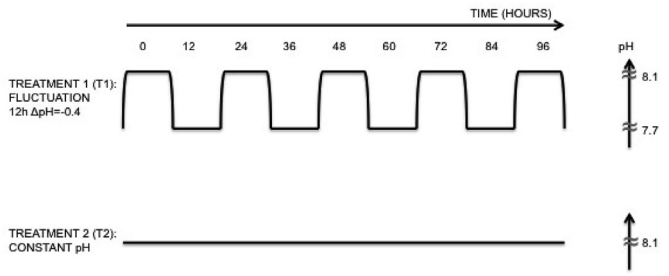

Fig. 1. Diagram representing the seawater pH fluctuation frequencies used during the *Paracentrotus lividus* larvae experiment: (T1) 12 h fluctuation from 7.7 to 8.1 units of pH and (T2) constant pH treatment of 8.1 units of pH.

When larvae within the cultures reached the competent stage and they were observed to have tube feet extending from the rudiment (Cameron and Hinegardner, 1974), a settlement experiment was carried out. The settlement assays were conducted using 1L containers. Ten competent larvae were set in each beaker with glass plates (9 cm diameter) with natural biofilm to induce settlement, corresponding to 6 different settlement treatments (n = 3 for each treatment): (t1) larvae that came from the 12 h fluctuating treatment and continued in this pH regimen; (t2) larvae with the same conditions that in the previous treatment but with the addition of a covering of macroalgae (*Cystoseira abies-marina*) placed at the bottom of the beaker (a layer coverage occupying half the diameter of the glass plate); (t3) larvae that came from the 12 h fluctuating treatment and changed to constant pH treatment of 8.1 units; (t4) larvae that came from constant pH treatment (8.1 units) and changed to 12 h fluctuating treatment; (t5) The same conditions that in treatment t4 but with the addition of a covering of algae (*Cystoseira abies-marina*) at the bottom of the beaker; (t6) larvae that came from constant pH treatment (8.1 units) and remained in these conditions.

The 50% treatment seawater was replaced every 24 h, adding seawater with the right pH, in order to create the fluctuation conditions, during which time any dead larvae were removed, minimising changes in water chemistry. This experiment was conducted without feeding and aeration.

To keep constant temperature conditions thermostat coolers (EHEIM AQUATICS, 50 W) were used. All experiments were conducted with FSW purified within a recirculating system provided with DRYDEN AQUA active filter media (AFM) bio-crystals; 50 µm, 10 µm and 1 µm UNICEL polyamide paper filters, as well as a UV-C AQUAEL 11W filter. FSW was prepared with the proper temperature and pH conditions for each treatment before using it.

2.3. Biological measurements

Larvae were sampled for a period of a month to quantify survival, growth, development and settlement. In each replicate beaker, three 2 mL aliquots were collected every second day and larvae were counted to estimate the density. Ten larvae in each replicate beaker were photographed every sampling day using a digital camera mounted on a binocular microscope. Several parameters were measured on each larva: body length (BL), post-oral arm length (PL) and stomach diameter (S1 & S2) (Fig. 2). Stomach volume (SV) was calculated as $SV = 4/3\ \pi\ ((S1+S2)/4)^3$ (Dorey et al., 2013).

After 7 days of competent larvae addition to the experimental containers for settlement experiments, the numbers of swimming, dead and settled postlarvae were counted in each treatment.

2.4. Data analyses

In order to assess the effects of pH fluctuation on larval survival, data were analysed by means of a two-way permutational analysis of variance (PERANOVA) (Anderson, 2001). A two-way design was conducted with pH (2 levels) and time (13 levels) as fixed factors.

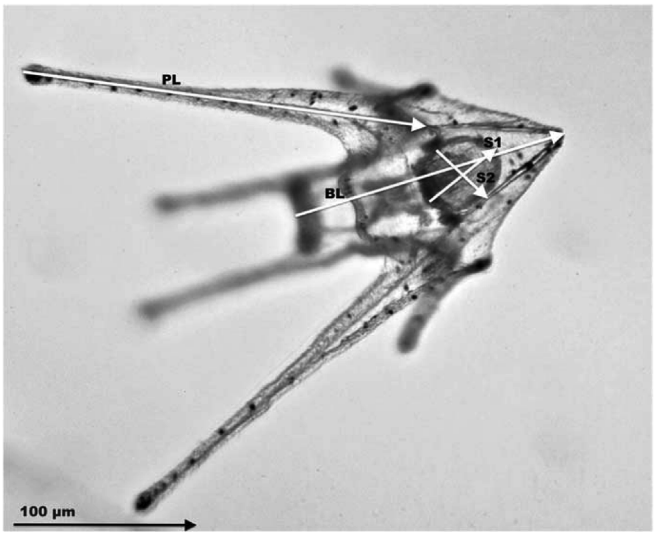

Fig. 2. Morphometric measurements taken for each sea urchin *Paracentrotus lividus* larvae: body length (BL), post oral arm length (PL) and stomach diameters (S1 & S2).

To evaluate the effect on morphometric measurements (BL, PL), two-way permutational multivariate analysis of variance (PERMANOVA) was performed. A two-way design was carried out with factors pH (2 levels) and time (13 levels) as fixed factors. To assess the impact on stomach volume (SV), a two-way permutational ANOVA was performed, using the same two-way design.

The effect of different settlement treatments (pH fluctuation frequencies and/or presence or lack of algae cover) on settlement and postlarvae test diameter was analysed by means of one-way permutational analyses of variance (PERANOVAs). One-way designs were conducted with factor settlement treatment (6 levels) used as fixed factor.

Euclidean distances were used for all analyses of variance and respective significant terms were examined using *a posteriori* pairwise comparisons by permutations (Anderson, 2001). If there were not enough possible permutations for a reasonable test, corrected p-values were obtained with Monte Carlo random draws from the asymptotic permutation distribution. Principal Coordinates Analysis (PCO) ordinations of morphometric measurements data were used to identify similarities between observations. All statistical analyses were carried out using PRIMER 6 & PERMANOVA + v. 1.0.1 software.

3. Results

Physico-chemical parameters of seawater during the larvae experiment are given in Table 1. Carbon dioxide partial pressure (pCO_2) was increased at the fluctuating pH treatment, while saturation levels of calcite (Ω_c) and aragonite (Ω_a) were decreased. However, seawater was not saturated in respect to calcite or aragonite (Ω_c, $\Omega a < 1$) in either treatments. The daily pH fluctuation in each experimental treatment in the larvae experiment is shown in Fig. 3.

No effect on survival was detected between the different pH frequencies, neither in the interaction of factors 'pH x Time' (Table 2), meaning that there were not different responses of larval survival at each treatment of pH tested with time (Fig. 4). The analysis only detected a clear effect of time on decreasing larval survival regardless of the pH regimen tested (Table 2, Fig. 4).

Results of the PERMANOVA analysing the morphometric measurements of the studied species showed a significant interaction of factors 'pH x Time' (Table 3A), indicating that the influence of pH on the BL and PL varied significantly across time during the larval development

Table 1
Physico-chemical seawater parameters for (A) the 12 h pH fluctuation treatment from 7.7 to 8.1 units, and (B) the constant treatment of 8.1 units of pH tested for larvae of *Paracentrotus lividus*. T: seawater temperature (mean ± SD), S: salinity (mean ± SD), pH: seawater pH (mean ± SD), pCO_2: CO_2 partial pressure, TA: Total alkalinity (mean ± SD), Ω_c: Saturation level of calcite, Ω_a: Saturation level of aragonite.

	A. Fluctuating treatment		B. Constant treatment	
	0 h	12 h	0 h	12 h
T (n = 23)	19.04 ± 2.18	19.08 ± 0.22	18.80 ± 0.22	19.02 ± 0.18
S (n = 23)	36.57 ± 2.18	36.82 ± 0.09	36.87 ± 0.08	36.85 ± ± 0.08
pH (n = 23)	7.67 ± 0.05	8.00 ± 0.04	8.02 ± 0.02	8.02 ± 0.02
pCO_2	1038.10		415.5	
TA (n = 3)	2303.08 ± 53.01		2315.43 ± 38.57	
Ωc	2.12		4.24	
Ωa	1.38		2.75	

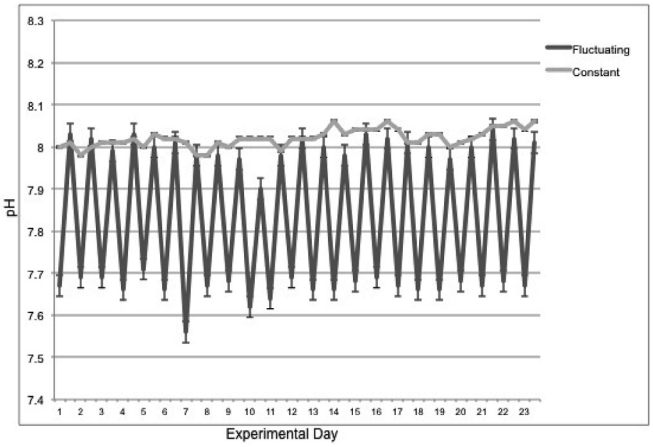

Fig. 3. Daily measurements of seawater pH (mean ± SD) recorded in both fluctuating and constant experimental treatments used for the larvae experiment of the sea urchin *Paracentrotus lividus*.

Table 2
Results of the two-way permutational ANOVA analyzing larval survival of the sea urchin *Paracentrotus lividus* in laboratory experiments, testing the effects of seawater pH daily fluctuations by means of comparing a 12 h pH fluctuation treatment from 7.7 to 8.1 units, and a constant treatment of 8.1 units of pH during the larval cycle of the species. The factors included in the model are: pH and Ti: Time.

Source of variation	df	SS	MS	Pseudo-F	P (perm)
pH	1	6.35E-2	6.35E-2	7.20E-2	0.800
Ti	13	646.92	49.76	56.44	0.001
pH *Ti	13	10.02	0.77	0.87	0.587

cycle in different manners in both pH treatments. However, a *posteriori* pairwise tests showed that in the majority of the sampling times there were not significant differences between treatments on larvae growth (Supplementary material 1; Fig. 5).

Stomach volume results revealed a significant interaction of factors 'pH x Time' (Table 3B), indicating that the influence of pH on the SV varied significantly across time during *P.lividus* larval development cycle. Nevertheless, pairwise analyses only showed significant differences between treatments in two of the thirteen sampling days and marginally significant differences in two additional sampling times (Supplementary material 2; Fig. 6).

With regard to developmental dynamics, larvae with 6 arms appeared at day 11 of experiment in treatment 1 (fluctuating), and two days later on treatment 2 (constant). The same delay was observed with

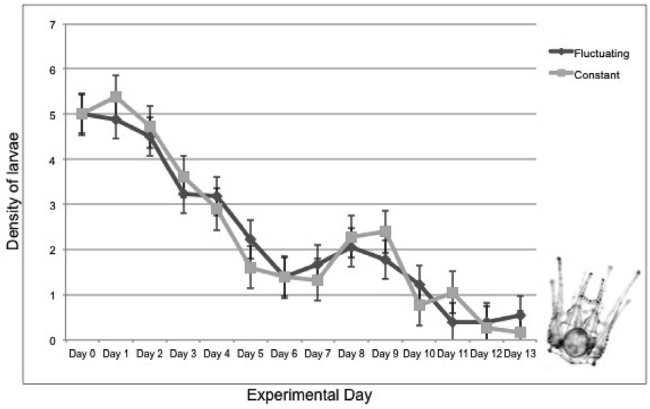

Fig. 4. Density of larvae of the sea urchin *Paracentrotus lividus* (mean ± SD) during the course of laboratory experiments testing the effects of contrasting treatments of pH frequencies: 12 h fluctuation treatment from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH.

8 arms larvae and the competent stage (Fig. 7).

Physico-chemical parameters of seawater during the settlement experiment are given in Table 4. Carbon dioxide partial pressure (pCO_2) was increased at the fluctuating pH treatments (t1, t2, t4 and t5), while saturation levels of calcite (Ω_c) and aragonite (Ω_a) were decreased. However, seawater was not saturated in respect to calcite or aragonite (Ω_c, $\Omega a < 1$) in either treatment. The daily pH fluctuation in each experimental treatment in settlement experiment is shown in Fig. 8.

Postlarvae settlement showed a significant effect of factor pH (Table 5A), showing that settlement of the species varied with each treatment of pH. *A posteriori* pairwise test revealed that treatments t2 and t5, which used an addition of a covering of algae (*Cystoseira abies-marina*), had a similar response on settlement and was significantly different from the other treatments (Supplementary material 3; Fig. 9).

When analysing postlarvae test diameter, not significant effect of pH treatments used in the experiment were detected (Table 5B). Postlarvae sea urchins in t2 showed a mean test diameter of 0.326 mm (SD ± 0.020), and in t5, it showed a mean diameter of 0.328(SD ± 0.030).

4. Discussion

The present study explored the sensitivities of *P. lividus* larvae to different pH environments covering the current regional variability, showing that sea urchin larvae are able to develop normally in both daily fluctuating and constant pH environments. However, larvae performance was slightly enhanced by a moderated pH fluctuation.

We did not found clear effects of the pH fluctuation frequencies tested in the laboratory on the survival, growth or stomach volume of *P. lividus* larvae. Only one study has also explored natural pH variability on larvae development with similar results on the intertidal species *Strongylocentrotus droebachiensis* where no effect on the same larvae performance variables was found between the different current frequencies (from 7.7 to 8.1 vs constant treatment of 8.1) (Peeters, 2013). Although responses to environmental factors seem to be highly species-specific, even in closely related taxa (Wittmann and Pörtner, 2013), most larvae of echinoderms are robust under moderated changes in seawater pH with respect to survival and growth, despite growth is usually delayed by pH values deviating from the natural variability (see reviews by Byrne et al. 2013a,b; Dupont and Thorndyke, 2013). It is thought that the capacity for extracellular acid-base regulation is key in determining a species' ability to cope with high environmental variability (Pörtner, 2008; Melzner et al., 2009; Calosi et al., 2013). On the contrary to a previous view that echinoids have limited regulatory ability (Boolootian, 1966; Binyon, 1972), recent studies indicate the

Table 3
Results of the two-way (A) PERMANOVA analyzing body length and post oral arm length; and (B) permutational ANOVA testing stomach volume (SV) of larvae of the sea urchin *Paracentrotus lividus* in laboratory experiments testing the effects of seawater pH daily fluctuation, comparing a 12 h pH fluctuation treatment (7.7–8.1 units), and a constant treatment (8.1 units) during the larval cycle of the species. In the respective models the factors included are: pH and Ti: Time.

Source of variation	A. Morphometric measurements					B. Stomach volume				
	df	SS	MS	Pseudo-F	P (perm)	df	SS	MS	Pseudo-F	P (perm)
pH	1	1.87E-2	1.87E-2	1.01	0.340	1	1.87E-2	1.87E-2	1.01	0.304
Ti	12	17.54	1.46	78.71	0.001	12	17.54	1.46	78.71	0.001
pH *Ti	12	0.61	5.05E-2	2.72	0.001	12	0.61	5.05E-2	2.72	0.002

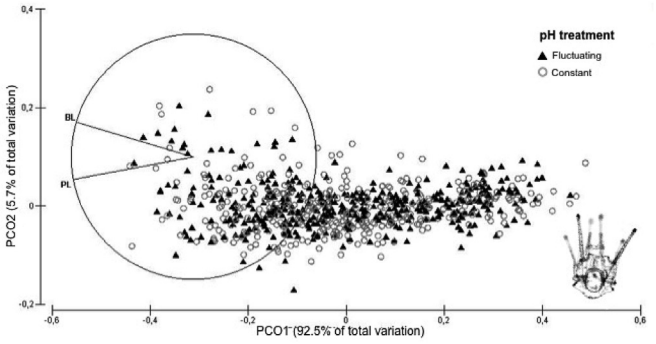

Fig. 5. PCO ordinations showing the effect of different treatments of pH frequencies (12 h fluctuation treatment from 7.7 to 8.1 units of pH, and constant pH treatment of 8.1 units of pH) on *Paracentrotus lividus* larvae development. Vectors represent studied variables. BL: Body Length, PL: Post oral arm Length. Percentages of variation explained by each of the axes are given in brackets.

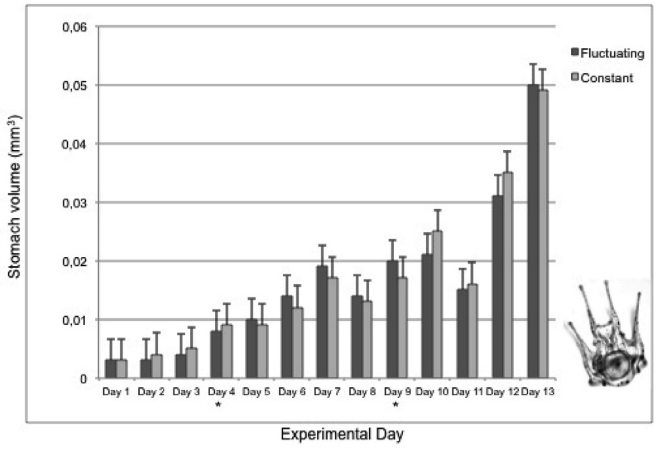

Fig. 6. Stomach volume (mm^3) of larvae of the sea urchin *Paracentrotus lividus* (mean ± SD) at laboratory experiments testing the effects of different treatments of pH frequencies: fluctuating (from 7.7 to 8.1 units of pH) and constant (≈8.1). * Significant differences.

presence of a suite of responses to low pH conditions in some sea urchin species (Spicer et al., 2011; Stumpp et al., 2011; Catarino et al., 2012; Calosi et al., 2013). In this sense, species success may in part be determined by their homeostatic abilities and associated energy cost (Stumpp et al., 2011; Catarino et al., 2012; Calosi et al., 2013). Ion regulation in echinoids is species-specific (Binyon, 1966). This ability is even more important in sea urchins inhabiting coastal or shallow water environments where sharp environmental oscillations occur. The ability to buffer such fluctuations, even if partially, can be an adaptive feature that allows organisms to cope with environmental stresses (Catarino et al., 2012). A recent study suggests that adults of *P. lividus* have a higher capacity than other sea urchin species to compensate its internal fluid pH in cases of moderate hypercapnia of seawater. This fact is

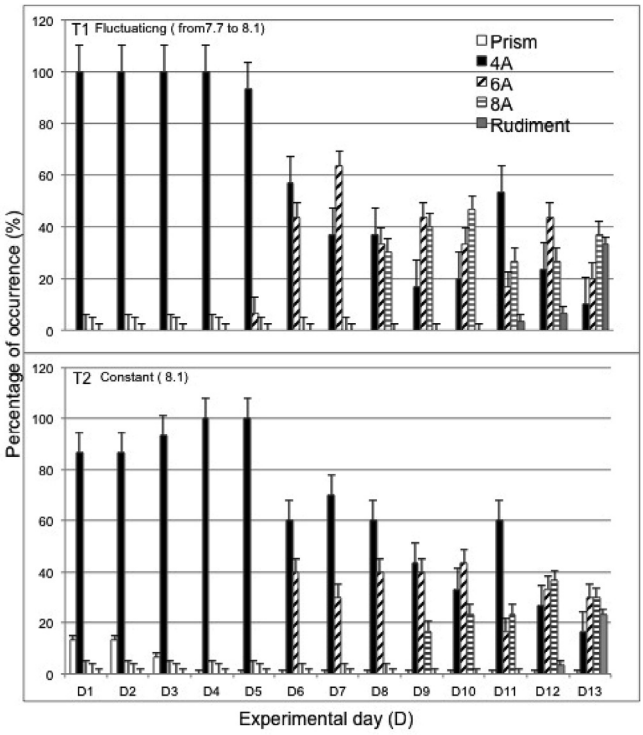

Fig. 7. Developmental dynamics of *Paracentrotus lividus* larvae during the experiment showing the percentage of occurrence of the different larval stages (prism, 4 arms (4A), 6 arms (6A), 8 arms (8A) and rudiment) in the pH treatments tested: (T1) 12 h fluctuation from 7.7 to 8.1 units of pH and (T2) constant pH treatment of 8.1 units of pH.

likely related to an acclimation ability of the species that allows it to cope with intertidal seawater parameters fluctuations (Catarino et al., 2012). In this sense, *P. lividus*'s adaptation potential, related to the great variety of habitats and environments that this species cope by crossing latitudes (Moulin et al., 2011), appears to play an important role in the species performance across a range of current environmental variability and it could be an important advantage in the struggle for survival in future climate change scenarios (García et al., 2018).

Differences were detected with regard to sea urchin developmental dynamics. In this sense, larvae with 6 arms appeared at day 11 of experiment in fluctuating treatment (T1), while in constant treatment (T2) it appeared two days later. The same delay was observed with 8 arms larvae and the competent stage. A delay in developmental dynamics has been reported for other species when the larvae suffer stress of a low constant pH treatment with values expected for the end of the century (see review by Dupont and Thorndyke, 2013). However, we found the delay in the constant treatment, corresponding to pH values that have typically been used as control treatments in OA experiments (see review by Byrne et al. 2013a,b; Dupont and Thorndyke, 2013). These results suggest that *P. lividus* larvae would perform better in environments with a moderate variability of seawater pH. A moderated

Table 4
Physico-chemical seawater parameters for each experimental treatment testing the effects of seawater pH daily fluctuation on *Paracentrotus lividus* settlement. Experimental treatments included larvae that were raised at (t1) a 12 h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoseira abies marina*), (t3) the 12 h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 12 h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions. T: seawater temperature (mean ± SD), S: salinity (mean ± SD), pH: seawater pH (mean ± SD), pCO_2: CO_2 partial pressure, TA: Total alkalinity, Ω_c: Saturation level of calcite, Ω_a: Saturation level of aragonite.

	t1	t2		t3		t4		t5		t6	
	0 h	12 h	0 h	12 h	0 h	12 h	0 h	12 h	0 h	12 h	0 h	12 h
T (n = 7)	19.04 ± 0.30	19.13 ± 0.30	19.05 ± 0.30	19.03 ± 0.26	19.12 ± 0.32	19.12 ± 0.32	19.00 ± 0.26	19.02 ± 0.26	19.00 ± 0.27	19.10 ± 0.28	19.14 ± 0.27	19.10 ± 0.28
S (n = 7)	37.01 ± 0.16	36.97 ± 0.12	36.78 ± 1.31	36.95 ± 0.12	37.02 ± 0.14	36.99 ± 0.13	37.03 ± 0.13	36.98 ± 0.11	37.06 ± 0.15	36.94 ± 0.11	37.02 ± 0.25	36.98 ± 0.13
pH (n = 7)	7.67 ± 0.04	8.00 ± 0.04	7.69 ± 0.02	8.00 ± 0.03	8.11 ± 0.02	8.12 ± 0.01	7.69 ± 0.03	8.00 ± 0.04	7.69 ± 0.03	8.01 ± 0.03	8.11 ± 0.02	8.12 ± 0.02
pCO_2	1062.50		1018.40		319.00		1015.30		1012.70		332.00	
TA (n = 1)	2360.88		2377.46		2289.75		2373.47		2367.71		2378.70	
Ωc	2.19		2.29		4.99		2.29		2.28		5.20	
Ωa	1.42		1.49		3.24		1.49		1.48		3.38	

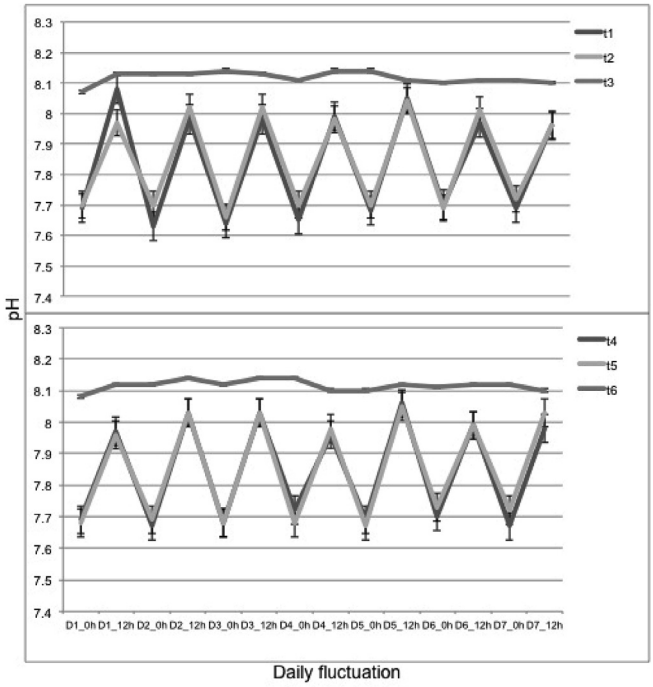

Fig. 8. Daily measurements of seawater pH (mean ± SD) recorded in each experimental treatment used for the settlement experiment of *Paracentrotus lividus*: larvae that were raised at (t1) a 12 h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoseira abies marina*), (t3) the 12 h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 12 h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions.

Table 5
Results of the one-way permutational ANOVA analyzing (A) settlement, and (B) test diameter of *Paracentrotus lividus* postlarvae, testing the effects of seawater pH daily fluctuations. The factor included in each model is settlement treatment.

A. Settlement					
Source of variation	df	SS	MS	Pseudo-F	P (perm)
Settlement treatment	5	209.89	41.98	7.48	0.003
B. Diameter					
Source of variation	df	SS	MS	Pseudo-F	P (perm)
Settlement treatment	1	2.86E-5	2.86E-5	3.04E-2	0.875

pH fluctuation such as the one used in our experiments, corresponding to the current variability of the parameter in nature, does not lead larvae near to its tolerance threshold. In this point, as the organism is not energy limited, thus a moderate decrease in pH could increase metabolism and lead a positive response. This shift in the energy budget does not limit the scope for growth in *P. lividus*, suggesting that the species is far from its tolerance threshold (Pörtner and Farrell, 2008; Stumpp et al., 2011). The repeated exposure to low pH conditions alternatively transient to high pH conditions could buffer the effects of acidification relieving physiological stress. This feature could potentially boost acclimation or adaptation to future more acidic environments (Hofmann et al., 2011).

Results of the settlement experiment showed very clear patterns since postlarvae settlement was only successful when a covering of algae was added to experimental units (t2 and t5), regardless of what was the frequency of pH that the larvae came from. Moreover, no differences in test diameter measurements were detected between juveniles settled at both fluctuating and constant pH treatments. Some

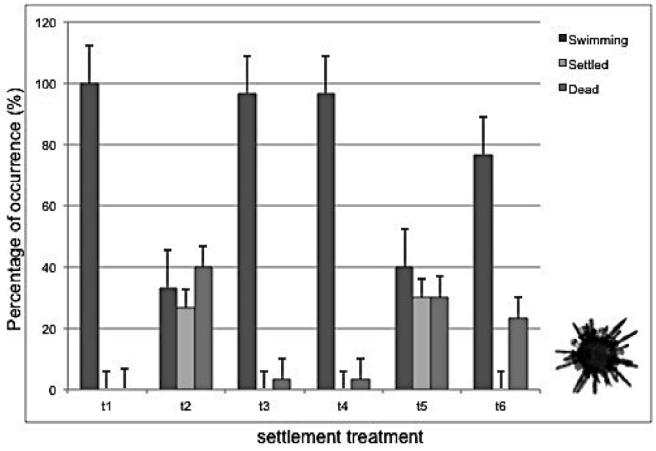

Fig. 9. Percentage of occurrence of swimming, settled and dead postlarvae at the end of the settlement experiment in the different treatments tested: larvae that were raised at (t1) a 12 h fluctuating treatment and continued in this frequency, (t2) the same conditions that the previous treatment but with the addition of a covering of algae (*Cystoseira abies marina*), (t3) the 12 h fluctuating treatment and changed to constant pH treatment (8.1 units), (t4) a constant pH treatment (8.1 units) and changed to 12 h fluctuating treatment, (t5) the same conditions that the previous treatment but with the addition of a covering of algae, and at (t6) the constant pH treatment (8.1 units) and remained in this conditions.

studies suggest that low seawater pH does not affect directly to the ability of larvae to settle, but indirectly by the alteration in the composition of inducers at low pH ranges (Webster et al., 2013). However, a recent study points out the addition of an alga inducer as a strong cue for settlers of the sea urchin *Strongylocentrotus droebachiensis* regardless of pH (Dorey, 2013). In our case, the daily oscillation in both fluctuating treatments (with absence or presence of alga cover, respectively) was similar. Thus, more than the fluctuation *per se*, our results suggest that the alga has a stronger component that induces postlarvae to settle.

In conclusion, *P. lividus* larvae development showed adaptive ecological strategies for inhabiting coastal areas covering present natural variability of seawater pH. The development of the species is surprisingly enhanced by a moderated pH fluctuation typical of intertidal environments that the sea urchin normally occupies. Considering the multidimensional range of environmental conditions that species' ecological niche possesses (Pörtner, 2002; Van Straalen, 2003), our results highlight the importance of considering the natural current variability of pH in the species' niche to a better understanding and forecast of future scenarios.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2018.04.012.

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